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Synthesis and Characterization of Sialylated Lactose- and Lactulose-Derived Oligosaccharides by *Trypanosoma cruzi* Trans-sialidase

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Supporting Information

ABSTRACT: Sialylated oligosaccharides contribute 12.6–21.9% of total free oligosaccharides in human milk (*h*MOS). These acidic *h*MOS possess prebiotic properties and display antiadhesive effects against pathogenic bacteria. Only limited amounts of sialylated *h*MOS are currently available. The aim of our work is to enzymatically synthesize sialylated oligosaccharides mimicking *h*MOS functionality. In this study, we tested mixtures of glucosylated-lactose (GL34), galactosylated-lactulose (LGOS), and galacto-oligosaccharide (Vivinal GOS) molecules, as trans-sialylation acceptor substrates. The recombinant trans-sialidase enzyme from *Trypanosoma cruzi* (TcTS) was used for enzymatic decoration, transferring (α 2 \rightarrow 3)-linked sialic acid from donor substrates to nonreducing terminal β -galactopyranosyl units of these acceptor substrates. The GL34 F2 2-Glc-Lac compound with an accessible terminal galactosyl residue was sialylated efficiently (conversion degree of 47.6%). TcTS sialylated at least 5 LGOS structures and 11 Vivinal GOS DP3–4 compounds. The newly synthesized sialylated oligosaccharides are interesting as potential *h*MOS mimics for applications in biomedical and functional-food products.

KEYWORDS: sialic acid, trans-sialidase, *Trypanosoma cruzi*, trans-sialylation, lactulose, galactooligosaccharides

INTRODUCTION

In human milk, free oligosaccharides constitute the third-most-abundant component after lactose and fat. Human milk oligosaccharides (*h*MOS) represent lactose molecules elongated with *N*-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) with various glycosidic-linkage types.¹ Sialic acid can be coupled to galactose residues in *h*MOS with (α 2 \rightarrow 3) or (α 2 \rightarrow 6) linkages and to GlcNAc with (α 2 \rightarrow 6) linkages. These sialylated oligosaccharides contribute 12.6–21.9% of total *h*MOS.² There is increasing evidence for positive functional effects from this group of acidic oligosaccharides on human health.^{3–5} Specific *h*MOS structures, namely, disialyllacto-*N*-tetraose and 2'-fucosyllactose, prevented and reduced necrotizing enterocolitis (NEC) in neonatal rats and thus may be used to prevent NEC in formula-fed infants.^{6,7} Preventive effects against NEC were also observed with a Sia-GOS mixture, particularly with disialyllated GOS.^{7,8} 3'-Sialyllactose stimulates growth of various *Bifidobacterium* strains, including the infant-gut-related *Bifidobacterium longum* subsp. *infantis*.⁹ Sialylated oligosaccharides also prevent intestinal attachment of pathogens by acting as receptor analogues, competing with epithelial ligands for bacterial binding.^{10–14} Compared with human milk, free oligosaccharides in the milk of domesticated animals are much less abundant.¹⁵ Bovine milk, for instance, has only trace amounts of milk oligosaccharides.^{16,17} The natural scarcity of these highly bioactive sialylated oligosaccharides stimulated us to study the possible synthesis of mimics via trans-sialylation of β -galactose (β -Gal)-linked

compounds in various oligosaccharide mixtures. One example is the Vivinal GOS mixture that is commercially used in infant nutrition.^{18,19}

Recently, we have reported the enzymatic synthesis of two novel oligosaccharide mixtures (GL34 and LGOS) and their structural characterization.^{20,21} GL34 is a mixture of five (α 1 \rightarrow 2/3/4)-glucosylated lactose molecules, with a degree of polymerization (DP) of 3–4, synthesized from sucrose as the donor substrate by glucansucrases (Gtf180- Δ N and GtfA- Δ N) as biocatalysts (Scheme 1).²⁰ The GL34 mixture exhibits selective stimulatory effects on the growth of various strains of lactobacilli and bifidobacteria.²² LGOS is a mixture of (β 1 \rightarrow 3/4/6)-galactosylated lactulose molecules, with one or two galactosyl moieties, synthesized from lactulose as the donor and acceptor substrate by wild-type and mutant β -galactosidase enzymes from *Bacillus circulans* ATCC 31382 (Scheme 1).²¹ Previously, oligosaccharides derived from lactulose were shown to promote the growth of bifidobacteria and to exert beneficial effects on the digestive tract.^{23–26}

In view of the potential functional properties of these novel GL34 and LGOS oligosaccharides we decided to try and further develop their structures to better mimic acidic *h*MOS. In this study, trans-sialidase from *Trypanosoma cruzi* (TcTS)²⁷ was employed for the trans-sialylation of oligosaccharides in

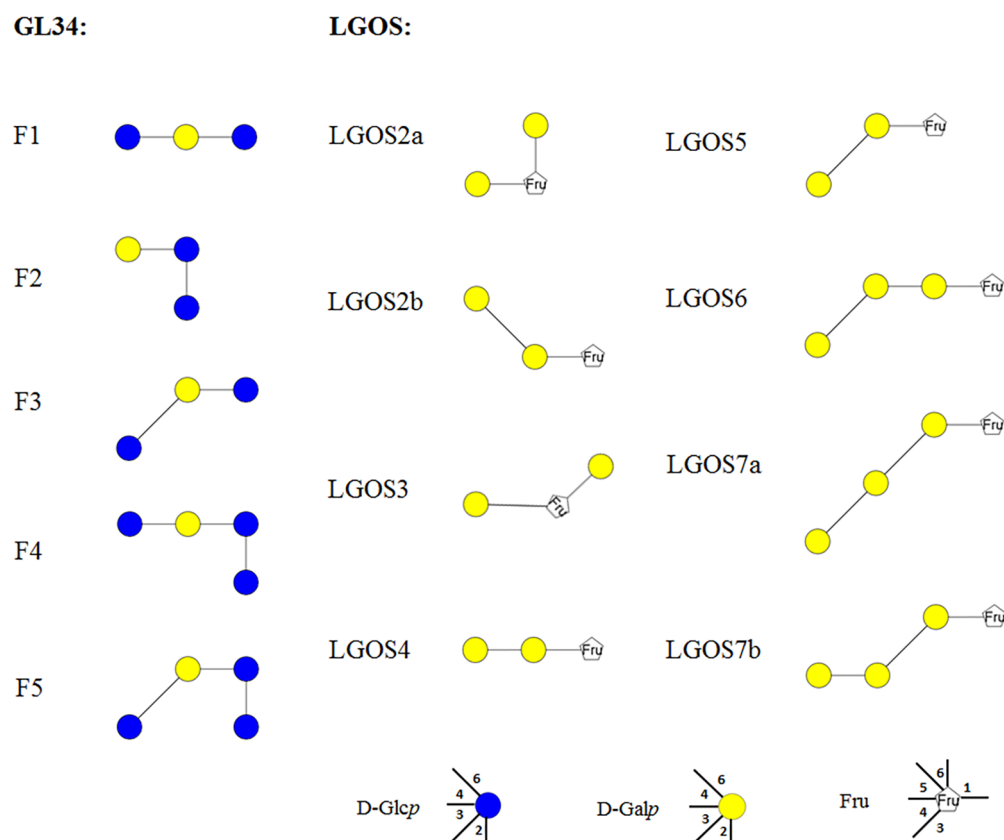
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Scheme 1. Schematic Presentation of All Structures in the GL34 Glucosylated-Lactose^a and LGOS Galactosylated-Lactulose^b Mixtures Used^{20,21}



^aLactose is galactosyl-glucose. ^bLactulose is galactosyl-fructose.

the GL34 and LGOS mixtures. Among trans-sialidases (EC 3.2.1.18), *T. cruzi* trans-sialidase is one of the best-studied enzymes.²⁷ It plays an important role in host-cell invasion and pathogenicity of *T. cruzi* because of its ability to scavenge and transfer sialic acid to the pathogen's extracellular mucins, thereby hiding the pathogen from the host immune system.^{28,29}

TcTS catalyzes trans-sialylation reactions via a ping-pong mechanism,³⁰ which starts with formation of a stable sialo-enzyme intermediate through a covalent bond with the nucleophile Tyr342.³¹ This is followed by transfer of the sialic acid to a β -Gal-linked acceptor substrate involving a nucleophilic attack of the hydroxyl group at C3 of this β -Gal.³⁰ When a suitable β -Gal-linked acceptor is absent, this enzyme catalyzes a hydrolysis reaction and sialic acid is released.³² In case of TcTS, sialyl transfer is catalyzed with much greater efficiency than hydrolysis.³³ TcTS can use glycoproteins or oligosaccharides as acceptor substrates, but it only uses compounds possessing sialic acid ($\alpha 2 \rightarrow 3$)-linked to a terminal β -Gal as donor substrates.³³ In previous work, we showed that TcTS catalyzes the transfer of sialic acid from a κ -casein-derived glyco-macropptide (GMP) donor substrate to galacto-oligosaccharides (GOS).^{8,34} However, a detailed analysis of these monosialylated and disialylated GOS structures was not performed. GMP is a byproduct of cheese manufacturing and contains a high levels of O-glycans, which carry Neu5Ac, including mainly Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)GalNAc and Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)[Neu5Ac($\alpha 2 \rightarrow 6$)]GalNAc, which can be used as donor substrates.^{35,36}

In this study, we used the GL34 and LGOS mixtures as acceptor substrates and GMP as the donor substrate. The negatively charged products were fractionated using Dowex 1 \times 8 chloride. Furthermore, we characterized the sialylated GOS structures that were synthesized in our previous work⁸ in more detail. The decorated GL34, GOS, and LGOS structures were identified using high-pH anion-exchange chromatography (HPAEC), and one-dimensional ¹H nuclear-magnetic-resonance spectroscopy (1D ¹H NMR spectroscopy).

MATERIALS AND METHODS

Chemicals and Materials. Bovine κ -casein-derived glyco-macropptide (GMP) was provided by the FrieslandCampina Innovation Center. *N*-Acetylneuraminic acid (Neu5Ac), 2-O-(4-methylumbelliferyl)- α -*N*-acetylneuraminic acid (4MU-Neu5Ac), and *N*-acetylneuraminyl($\alpha 2 \rightarrow 3$)lactose (3'-SL) were obtained from Carbo-synth Ltd. Neuraminidase from *Clostridium perfringens* was obtained from Roche. Synthesis of glucosylated-lactose compounds (GL34),²⁰ galactosylated-lactulose compounds (LGOS),²¹ and sialylated Vivinal GOS (DP3 and DP4) compounds⁸ has been reported previously.

TcTS Expression and Purification. *Escherichia coli* BL21 (DE3) (Invitrogen) was used as the host for the expression of the trans-sialidase from *Trypanosoma cruzi*. Precultures of *E. coli* BL21 (DE3) harboring pTrcTS611/2 were cultured overnight at 30 °C.^{37,34} Terrific broth (TrB) with 12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol, and 100 μ g mL⁻¹ ampicillin was inoculated with 1% preculture at 30 °C and 200 rpm. Expression of trans-sialidase was induced using 0.2 mM isopropyl β -D-thiogalactopyranoside (IPTG) when the cell density reached $A_{600} = 0.4$ – 0.6 . Cultivation was continued at 18 °C for 4 h. The cells were collected by centrifugation (10 min, 4 °C, 10 000g) and washed with 50 mM Tris-HCl buffer

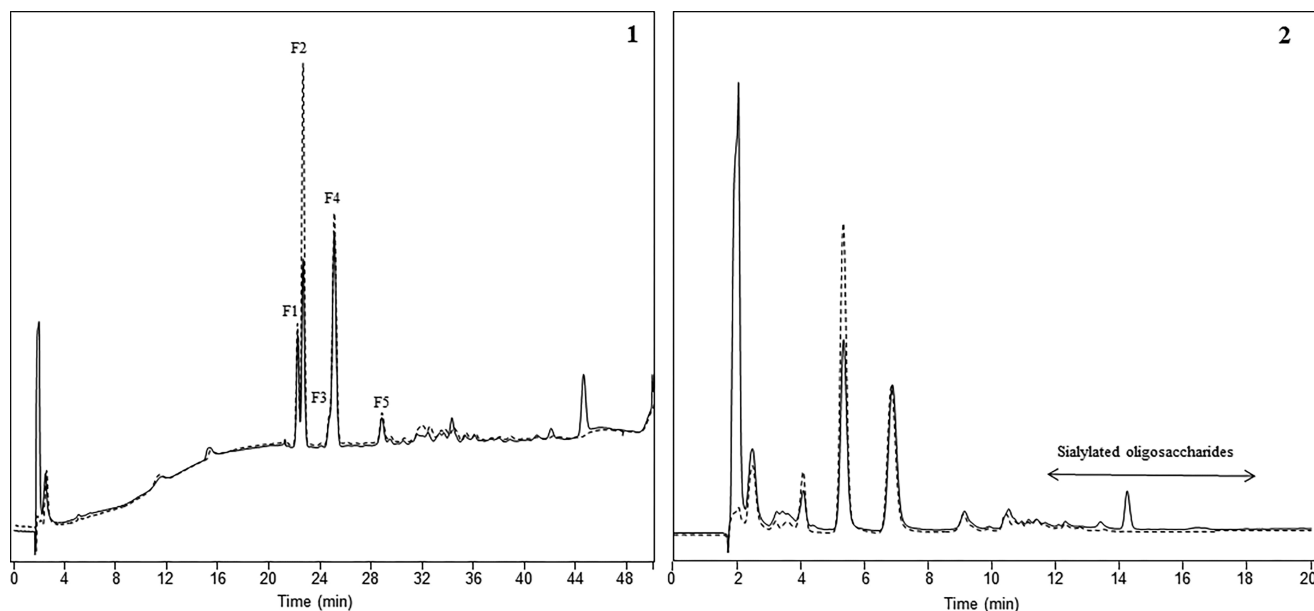


Figure 1. HPAEC-PAD profiles of compounds in the GL34/GMP/TcTS reaction mixture (incubation at 25 °C and pH 5.0, using 10 mM GL34) at $t = 0$ h (dotted line) and $t = 24$ h (solid line) using a CarboPac PA-1 column with gradients for (1) neutral oligosaccharides and (2) acidic oligosaccharides.

(pH 8.0). Cell resuspension by B-Per Tris solution (Thermo Scientific, Pierce) was followed by incubation at room temperature for 30 min. The trans-sialidase enzyme was purified with an HIS-Select Nickel Affinity Gel (Sigma). After 1.5 h of binding at 4 °C on a rotary shaker, the bound protein was consecutively washed with Tris-HCl buffer (50 mM, pH 8.0) containing NaCl (0.3 M) and imidazole (5 and 30 mM) prior to its elution with 300 mM imidazole in the same buffer. Purified TcTS enzyme was washed and concentrated in buffer Tris-HCl (pH 8.0) using a Millipore filter (50k).

Enzyme Incubations. TcTS (5 $\mu\text{g mL}^{-1}$) was incubated with various concentrations (1, 5, and 10 mM) of the GL34 mixture (average DP3) and 67.5 mg mL^{-1} GMP (corresponding to 5 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac)³⁴ in 50 mM sodium citrate buffer (pH 5.0) at 25 °C for 24 h. The reactions were stopped by heating at 65 °C for 10 min. Aliquots of 10 μL were diluted with 190 μL of DMSO (95%) for HPAEC-PAD analysis.

TcTS (5 $\mu\text{g mL}^{-1}$) was incubated with various concentrations (1, 5, 10, and 15 mM) of the LGOS mixture (average DP3) and 67.5 mg mL^{-1} GMP (corresponding to 5 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac)³⁴ in 50 mM sodium citrate buffer (pH 5.0) at 25 °C. TcTS (5 $\mu\text{g mL}^{-1}$) was added to the incubation mixture at $t = 0$ h and after each 24 h of incubation. Aliquots of 20 μL were sampled after 24, 48, and 72 h of incubation and mixed with 380 μL of DMSO (95%) for analysis by HPAEC-PAD profiling. The reactions were stopped by heating at 65 °C for 10 min.

Isolation of Negatively Charged Oligosaccharides by Dowex Chromatography. Dowex 1 \times 8 chloride (Cl^-) (Sigma-Aldrich) was packed in an Econo-column (1.5 \times 10 cm, Biorad) and activated with 10 column volumes (10 CV) of NaOH 2 M (at least 1 h contact time). Before injection of samples, the column was equilibrated with 10 CV of water. Elution of the sialylated oligosaccharides was performed at a flow-rate of 1 mL min^{-1} with Milli-Q water (MQ) and ammonium bicarbonate as eluents. After injection, unbound compounds were removed from the column by washing with MQ for 3 CV. Monosialylated and disialylated oligosaccharides were eluted with 3 CV of 50 and 400 mM ammonium bicarbonate, respectively. An extra elution step with 500 mM ammonium bicarbonate was used to wash off all remaining sialylated structures. The collected fractions were lyophilized.

Desialylation of Sialylated Oligosaccharides. Fractions of sialylated LGOS were treated with acetic acid (20%) for 1 h at room

temperature, which was followed by neutralization with 1 M NaOH. Desialylated fractions were desalted using Carbohydrate SPE columns.

Sialylated Vivinal GOS fractions of DP3 and DP4 were desialylated by incubation with 1 U mL^{-1} neuraminidase (Roche) in 0.1 M acetate buffer (pH 5.0) at 37 °C for 24 h.

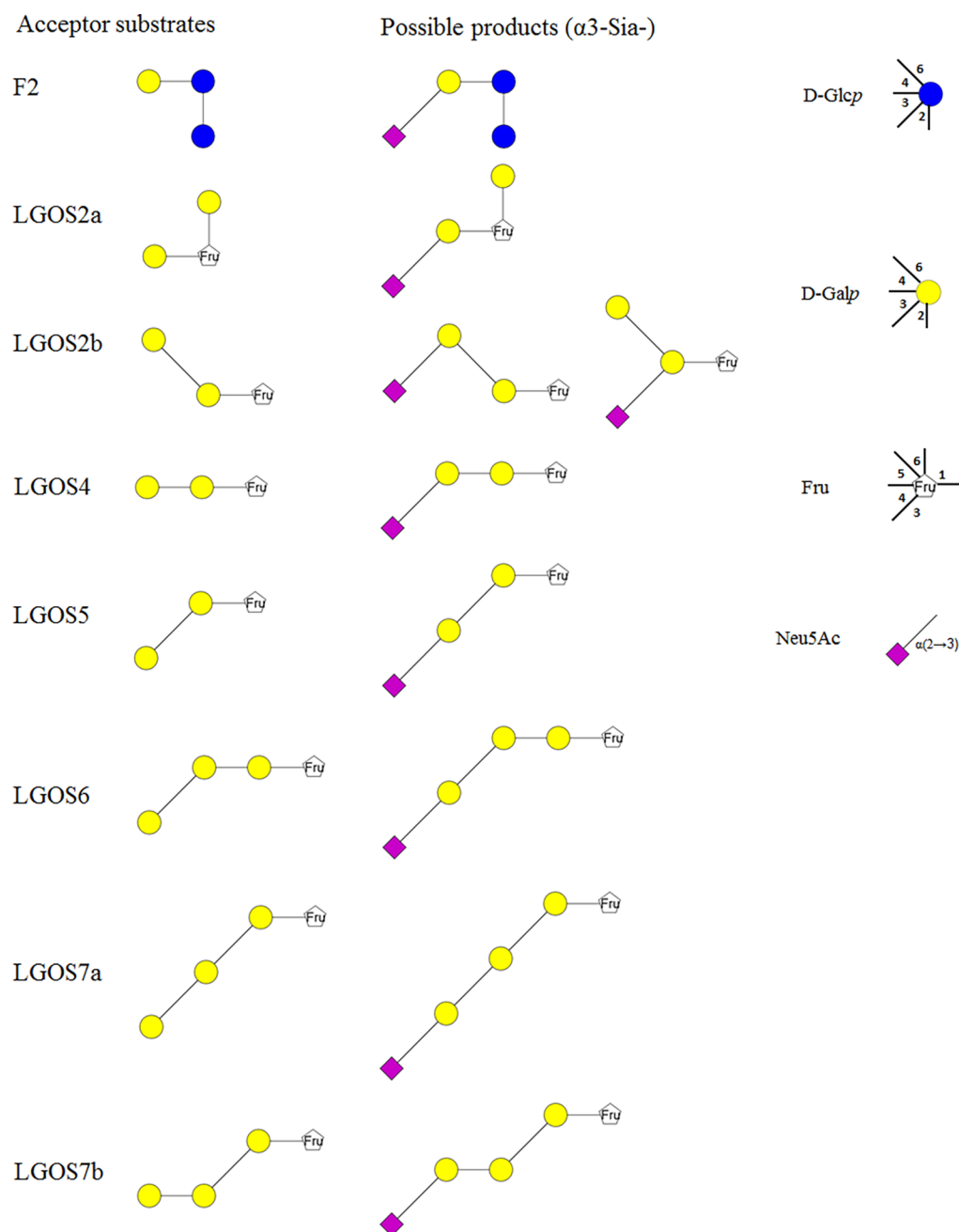
HPAEC-PAD Chromatography. Oligosaccharide mixtures were analyzed by HPAEC-PAD profiling on a Dionex ICS-3000 system (Thermo Scientific) equipped with a CarboPac PA-1 column (250 \times 2 mm, Dionex) and detected with a pulsed amperometric detector (PAD) using a gold working electrode. Eluting glycans were detected using a standard manufacturer's quadruple-potential waveform for detecting carbohydrates. Conversion of substrates into sialylated compounds was estimated by comparing converted peaks with unconverted peaks (F1 and F3 for GL34, Fru for LGOS) at different time points, calculating depletion of peaks. A gradient of 30 to 600 mM sodium acetate in 0.1 M NaOH (0.25 mL min^{-1}) was used for analytical separation of acidic oligosaccharides. Another complex gradient of eluents A (100 mM NaOH), B (600 mM NaOAc in 100 mM NaOH), C (Milli-Q water), and D (50 mM NaOAc) was used for profiling neutral oligosaccharide mixtures as previously described.²⁰

NMR Spectroscopy. Structures of the transferred compounds were determined by 1D ^1H NMR recorded at a probe temperature of 25 °C on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen). The samples were exchanged twice with D_2O (99.9 atom % D, Cambridge Isotope Laboratories, Inc.) with intermediate lyophilization and then dissolved in 0.65 mL of D_2O containing acetone as the internal standard ($\delta^1\text{H}$ 2.225 ppm). Data was recorded at 16k complex data points, and the HOD signal was suppressed using a WET1D pulse (500 MHz spectra). MestReNova 9.1.0 (Mestrelabs Research SL) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS AND DISCUSSION

Previously, *N*-acetylneuraminic acid (Neu5Ac) was determined to be a major component (>99%) of the 3.6% (w/w) sialic acid in GMP, in comparison with *N*-glycolylneuraminic acid (Neu5Gc).³⁴ A concentration of 67.5 mg mL^{-1} GMP, corresponding to 5 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac, was used as donor substrate for the incubations in this study. At this fixed concentration of GMP as a donor substrate, the

Scheme 2. Schematic Presentation of the ($\alpha 2 \rightarrow 3$)-Sialylation Product of F2 (GL34 Mixture) and Possible Structures of the 5–8 ($\alpha 2 \rightarrow 3$)-Sialylation Products of the LGOS Mixture



concentrations of the acceptor substrates necessary to obtain their maximal conversion degrees were determined. All the incubations were carried out under the optimal conditions for TcTS as previously reported (in 50 mM sodium citrate buffer pH 5.0 at 25 °C).^{34,38}

Sialylation of GL34 by TcTS. The mixture GL34 (average DP3) was incubated at concentrations of 1, 5, and 10 mM, with 67.5 mg mL⁻¹ GMP and 5 µg mL⁻¹ TcTS at 25 °C and pH 5.0 for 24 h. After incubation, the HPAEC-PAD profiles showed a new peak eluting at a retention time of ~14.5 min, which is in the retention area of negatively charged oligosaccharides in this gradient (Figure 1, spectrum 2).⁸ In the HPAEC-PAD profile of neutral oligosaccharides, only the F2-compound peak had a significantly decreased area (Figure 1, spectrum 1). These results suggested that F2 was used as an

acceptor substrate for trans-sialylation by TcTS. The signals of Neu5Ac($\alpha 2 \rightarrow 3$) (H-3e at δ 2.755 and H-3a at δ 1.795) were detected in the 1D ¹H NMR spectrum of the GL34 mixture after the trans-sialylation reaction (Figure S1). The presence of a new signal at δ 4.212 is fitting with the 3-substitution at the terminal galactosyl residue of F2 with Neu5Ac (Figure S1), confirming the synthesis of Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)[Glc($\alpha 1 \rightarrow 2$)]Glc (Scheme 2). On the basis of the HPAEC-PAD responses, the maximal conversion of F2 into the corresponding sialylated-F2 was observed with 10 mM GL34 and calculated as 47.6%, on the basis of reduction of the F2 peak. The data shows that only F2 was used as an acceptor substrate for trans-sialylation by TcTS. In the GL34 mixture, F2 is the only compound with an accessible β -Gal residue at a nonreducing terminal position (Scheme 1). TcTS was shown

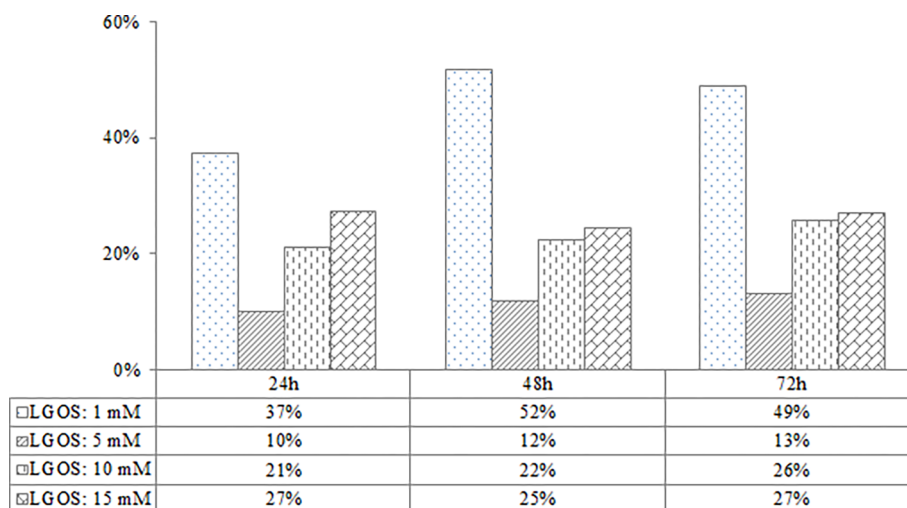


Figure 2. Conversion of the LGOS-mixture compounds into sialylated oligosaccharides at different concentrations of LGOS and various incubation times, with renewed addition of TcTS after each 24 h. Data obtained from HPAEC-PAD responses (in duplicate).

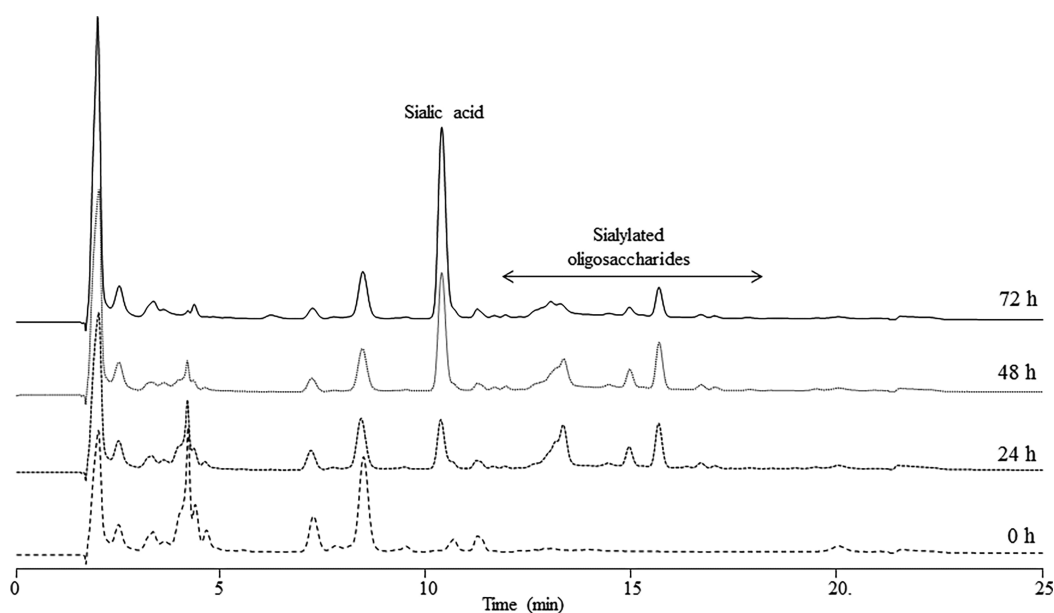


Figure 3. HPAEC-PAD profiles of compounds in the reaction mixtures of 1 mM LGOS, 5 mM GMP-derived Neu5Ac($\alpha 2 \rightarrow 3$), and 5 $\mu\text{g mL}^{-1}$ TcTS incubated at 25 °C and pH 5.0 for 0–72 h, with renewed addition of TcTS (5 $\mu\text{g mL}^{-1}$) after each 24 h of incubation. Neutral LGOS and negatively charged Sia-LGOS eluted at 2–12 and 12–18 min, respectively.

to also glycosylate internal β -Gal residues in specific structures (i.e., in a Gal($\beta 1 \rightarrow 6$)Gal epitope),^{27,30} but these are absent in GL34. F1 4'-Glc-Lac and F4 2,4'-Glc-Lac, the only other GL34 compounds with nonsubstituted OH-3 positions (on the internal galactose residue, Scheme 1),²⁰ were not used as acceptor substrates.

Sialylation of LGOS by TcTS. Various concentrations of the LGOS mixture (1, 5, 10, and 15 mM) were incubated with 67.5 mg mL^{-1} GMP as a donor substrate and with TcTS (5 $\mu\text{g mL}^{-1}$) at 25 °C and pH 5.0. Because of the relatively low stability of this trans-sialidase,³⁸ extra TcTS (5 $\mu\text{g mL}^{-1}$) was added to the incubation mixtures after every 24 h of incubation. The incubation experiments were followed over time, and the highest degree of LGOS conversion into sialylated LGOS was ~52% after 48 h with 1 mM LGOS mixture (Figure 2). Conversion of LGOS was estimated by following nonsialylated-LGOS-peak depletion over time,

assuming that all LGOS had similar response factors on the PAD detector. At this LGOS concentration, the conversion degree increased significantly from 37.4 to 52.0% when the incubation lasted from 24 to 48 h (Figure 2). In all cases, the GMP-derived Neu5Ac($\alpha 2 \rightarrow 3$) as the donor substrate was not completely utilized, with a maximal use of 80% when incubated with 15 mM LGOS for 24 h. Enhanced conversion degrees were not observed when incubating other concentrations of the LGOS mixture longer than 24 h despite renewed addition of TcTS (Figures S2–S4).

The HPAEC-PAD profiles of the incubation mixtures with 1 mM LGOS showed development of several new peaks over time (Figure 3). These new peaks eluted at retention times between 12–22 min, indicating synthesis of a complex mixture of sialylated LGOS. The negatively charged (Sia-LGOS) oligosaccharides were separated from the neutral (LGOS) oligosaccharides by Dowex 1 \times 8 (Cl^-) chromatography and

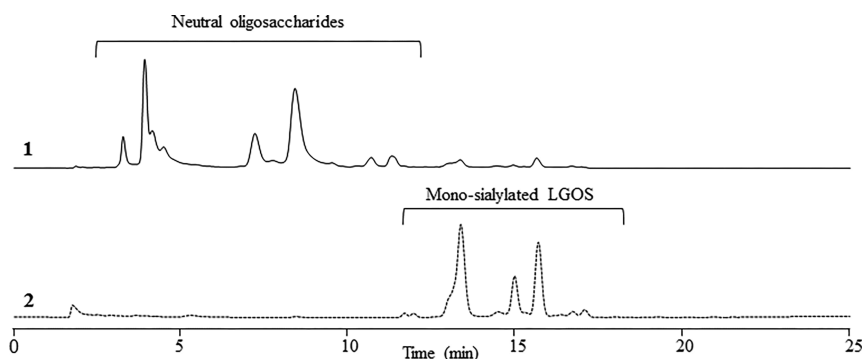


Figure 4. HPAEC-PAD profiles of the Dowex 1 \times 8 (Cl^-) chromatography fractions obtained by (1) MQ rinsing (neutral oligosaccharides) and (2) elution with 50 mM ammonium bicarbonate (Sia-LGOS). The reaction mixture of 1 mM LGOS, 5 mM GMP-derived Neu5Ac($\alpha 2 \rightarrow 3$), and 10 $\mu\text{g mL}^{-1}$ TcTS, incubated at 25 $^\circ\text{C}$ and pH 5.0 for 48 h, was used for Dowex chromatography.

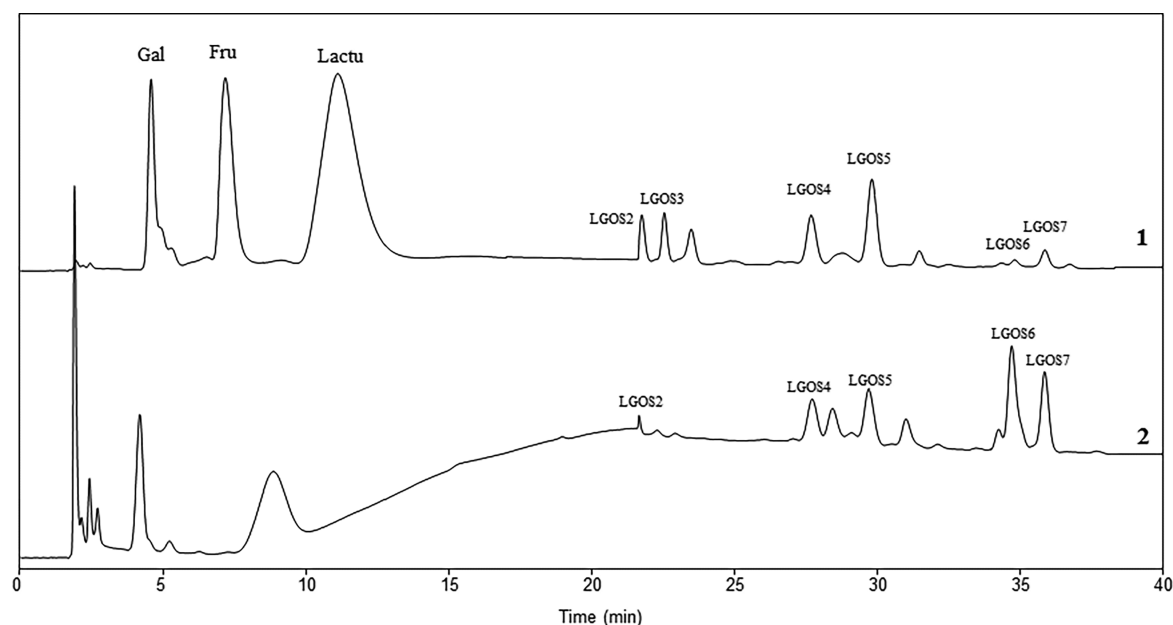


Figure 5. HPAEC-PAD profiles of compounds in (1) 1 mM LGOS incubation mixture before purification to remove mono- and disaccharides and (2) Sia-LGOS fraction after being desialylated by acetic acid (20%) treatment. Identified peaks are marked corresponding to the structures shown in Scheme 2. Peak 2 corresponds to the LGOS2a or LGOS2b structure. Peak 7 corresponds to the LGOS7a or LGOS7b structure.

then reanalyzed by HPAEC-PAD profiling (Figure 4). The neutral oligosaccharides in the unbound Dowex fraction eluted during the first 12 min in the HPAEC-PAD profile (Figure 4, spectrum 1). The Dowex fraction that eluted with 50 mM ammonium bicarbonate (Sia-LGOS) eluted between 12 and 18 min in the HPAEC-PAD profile (Figure 4, spectrum 2), fitting with monosialylated structures.⁸ The Dowex fraction containing disialylated structures was relatively minor, limiting possibilities for further characterization.

The 1D ^1H NMR spectrum (Figure S5) of the negatively charged fraction revealed signals at δ 2.760 and 1.803, which belong to the Neu5Ac H-3e and H-3a atoms, respectively, of the Neu5Ac($\alpha 2 \rightarrow 3$) residues.³⁹ These NMR-spectroscopy data confirmed the sialylation of LGOS by TcTS. To identify the compounds in the LGOS mixture that were decorated with Neu5Ac, desialylation of these sialylated-LGOS was carried out using 20% acetic acid.

Comparison of the HPAEC-PAD profiles of the desialylated fraction with that of the LGOS mixture allowed identification of at least five structures that were monosialylated by TcTS: LGOS2a or 2b, LGOS4, LGOS5, LGOS6, and LGOS7a or 7b

(Figure 5). In the LGOS profile structures, LGOS4 and LGOS5 are the major components. After sialylation and desialylation, the HPAEC-PAD profile showed LGOS6 and LGOS7 to be the predominant structures. In the LGOS mixture, LGOS6 is only a trace peak, but in the sialylated fraction, LGOS6 is the major structure. This indicated that the Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$) epitope is very favorable for sialylation. The LGOS7 peak consisted of two structures, one with a Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$) epitope and one with a Gal($\beta 1 \rightarrow 4$)Gal($\beta 1 \rightarrow 3$) epitope. Although it is not possible to distinguish between the two structures, it is likely that structure LGOS7a, with a terminal Gal($\beta 1 \rightarrow 3$) residue, is the mainly sialylated LGOS7 structure. This fits with previous results on galactosyl-lactose conversions, showing a much higher specificity constant ($k_{\text{cat}}/k_{\text{M}}$) for the transferase reaction to 3'-galactosyllactose than for the reactions to 4'-galactosyllactose and 6'-galactosyllactose.³⁴ Closer inspection of the 1D ^1H NMR profile of the Sia-LGOS fractions revealed the B f 4 signals at δ 4.200–4.211, which originate from the LGOS4, LGOS5, LGOS6, or LGOS7 structures (slightly shifted). This provided evidence for the presence of the LGOS4, LGOS5,

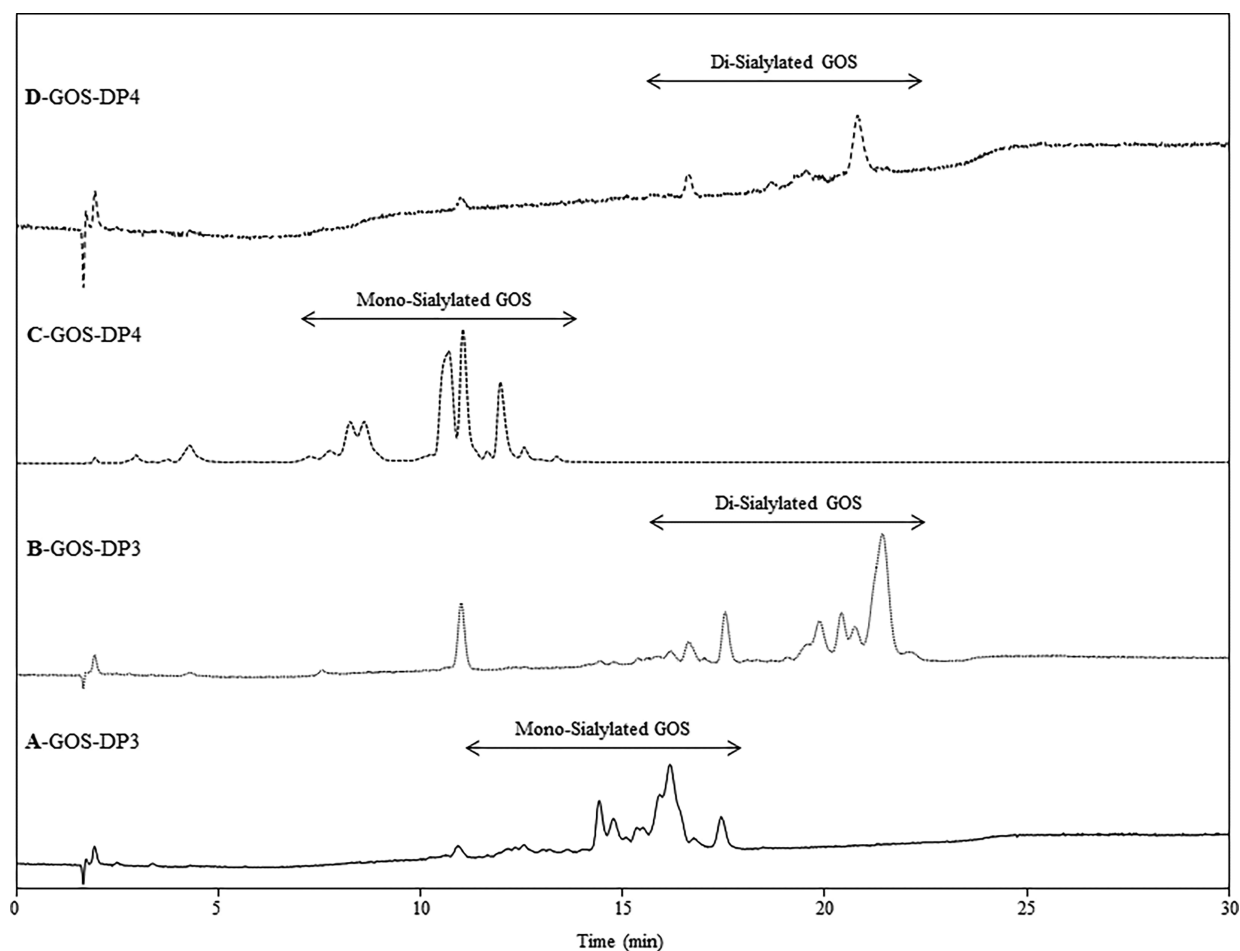


Figure 6. HPAEC-PAD profiles of the Sia-GOS (A,B) DP3 and (C,D) DP4 fractions. Mono- and disialylated-GOS peaks are marked.

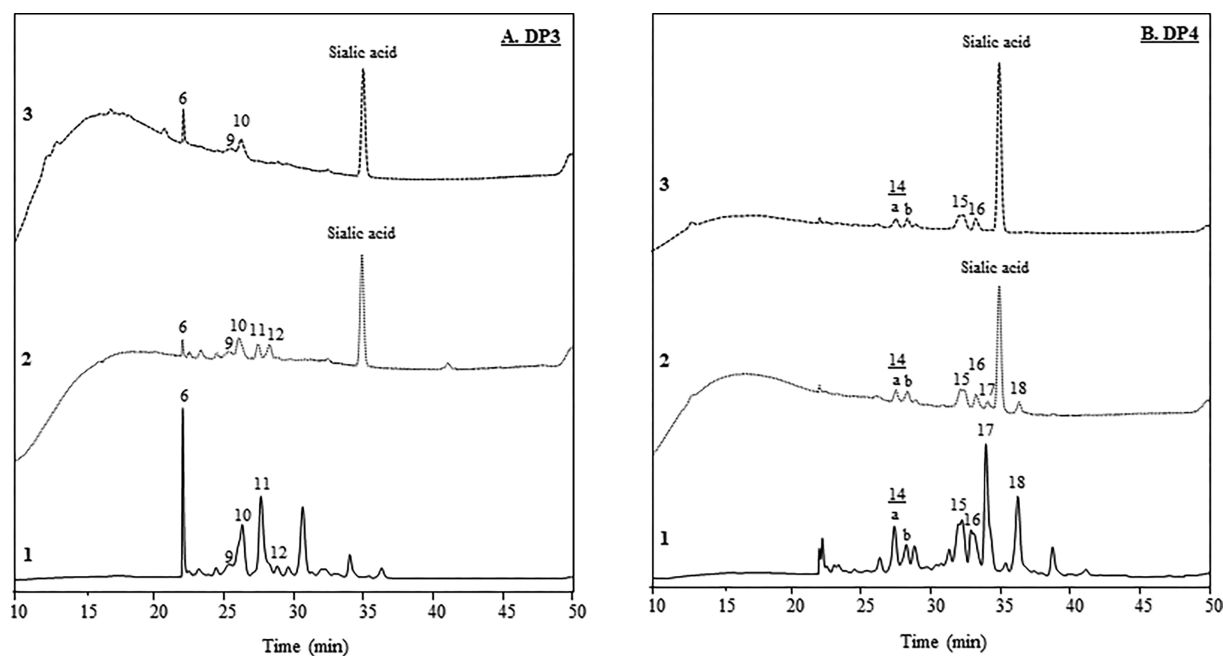
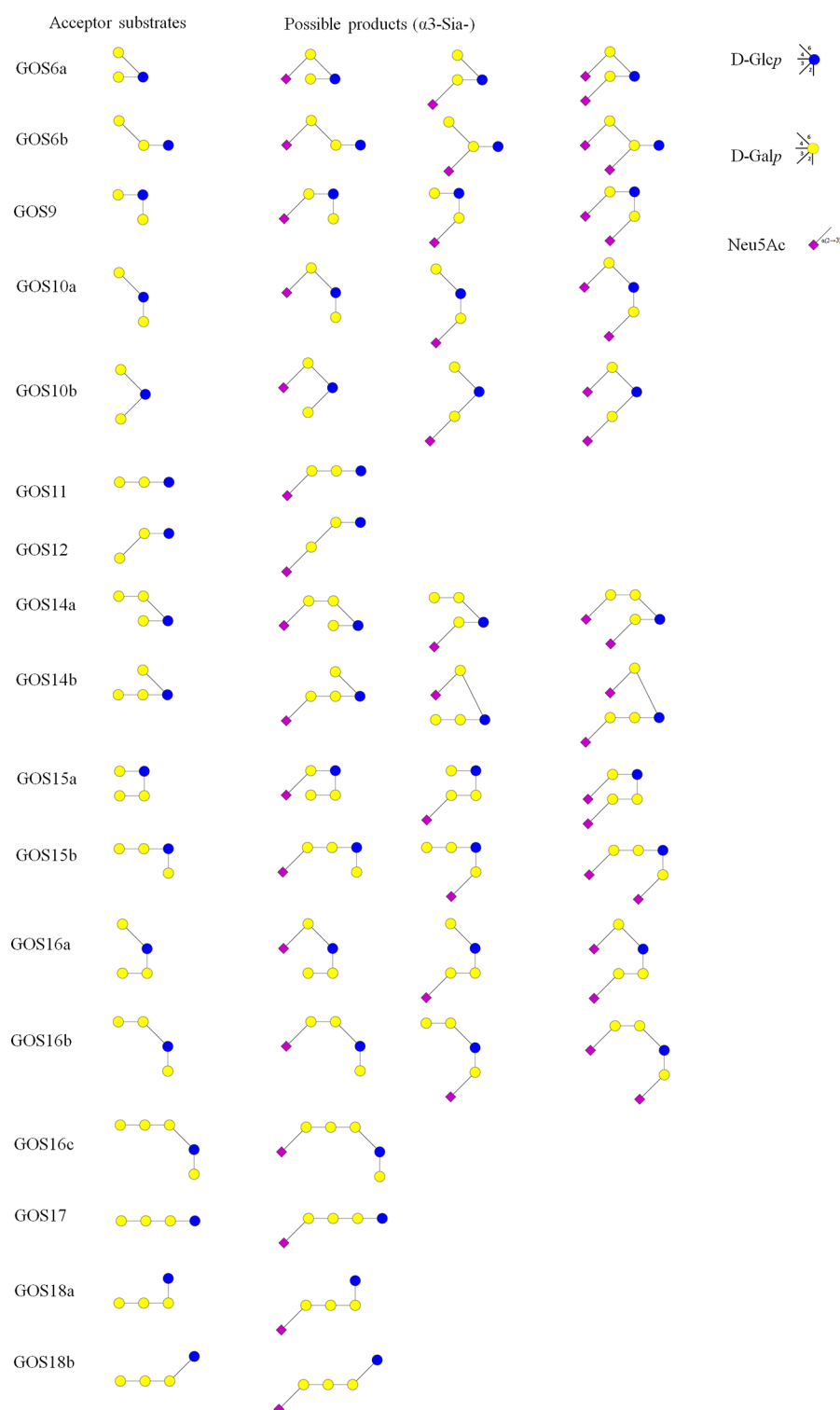


Figure 7. HPAEC-PAD profiles of compounds from sialylated-GOS fractions (A) DP3 and (B) DP4: (1) neutral GOS mixtures at corresponding DP, (2) mono-Sia-GOS, and (3) di-Sia-GOS fractions after being desialylated. Identified GOS peaks are marked with numbers as used by van Leeuwen et al.,⁴⁰ corresponding with those in Scheme 3. Peak 6 corresponds to GOS6a or 6b; peak 10 corresponds to GOS10a or 10b; peak 11 corresponds to GOS15a or 15b; peak 16 corresponds to GOS16a, 16b, or 16c; and peak 18 corresponds to GOS18a or 18b.

Scheme 3. Schematic Presentation of the Possible Structures of the 16–39 ($\alpha 2 \rightarrow 3$)-Sialylation Products of the Vivinal GOS DP3 and DP4 Fractions

LGOS6, or LGOS7 compounds in the Sia-LGOS mixture. Moreover, the ^1H NMR spectrum of this mixture showed anomeric C-1 signals (slightly shifted) at δ 4.694, 4.650, and 4.629 from the structures LGOS4–7.

In LGOS5 ($\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$) and LGOS7a ($\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$), the O-3 positions of the internal β -Gal residues are already substituted, only the terminal β -Gal residues of LGOS5 and LGOS7a are available

for ($\alpha 2 \rightarrow 3$)-linked decoration with Neu5Ac to yield the corresponding monosialylated oligosaccharides: Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)Fru and Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)Fru (Scheme 2). The structure of LGOS4 ($\text{Gal}(\beta 1 \rightarrow 4)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$) was only monosialylated by TcTS, although it also possesses a nonsubstituted O-3 of the internal β -Gal residue. This was also observed for the similar structure $\beta 4'$ -galactosyl-lactose, of which only the

terminal β -Gal residue was ($\alpha 2 \rightarrow 3$)-substituted with Neu5Ac.³⁴ The disialylated LGOS fraction was too minor to be elucidated. In the LGOS mixture, only LGOS1, LGOS2a, and LGOS3, each with two terminal β -Gal residues, as well as LGOS2b with an internal β -Gal residue ($\beta 1 \rightarrow 6$) linked with a terminal β -Gal residue, are likely disialylated.³⁴

Sialylation of GOS by TcTS. In our previous work, the Vivinal GOS DP3 and DP4 fractions were sialylated using TcTS.⁸ These sialylated mixtures were applied onto a Resource Q anion-exchange-chromatography column to obtain the separate monosialylated- and disialylated-GOS fractions, depending on the negative charges.⁸ The HPAEC-PAD profiles of the sialylated GOS DP3 fraction showed the presence of multiple mono-Sia-GOS compounds at retention times between 11 and 17 min and di-Sia-GOS compounds at retention times between 16 and 23 min (Figure 6A,B). Similarly, in the HPAEC-PAD profiles of the Sia-GOS DP4 fraction, the mono-Sia-GOS compounds eluted at retention times between 8 and 13 min, and the di-Sia-GOS compounds eluted at retention times between 17 and 23 min (Figure 6C,D). The Sia-GOS mixtures were incubated with neuraminidase from *Clostridium perfringens*, which prefers to hydrolyze ($\alpha 2 \rightarrow 3$)-linkages over ($\alpha 2 \rightarrow 6$)- and ($\alpha 2 \rightarrow 8$)-linked sialic acids, to remove the sialic acid groups attached to the GOS compounds. The HPAEC-PAD profiles of the desialylated fractions were compared with those of the Vivinal GOS mixture, which were previously annotated,^{40,41} in order to identify decorated structures (Figure 7A1,B1). In the GOS DP3 fraction, at least five structures were monosialylated, namely, GOS6a or 6b, GOS9, GOS10a or 10b, GOS11, and GOS12 (Figure 7A2). At least three of these seven structures were also found in the di-Sia-GOS fraction, namely, GOS6a or 6b, GOS9, and GOS10a or 10b (Figure 7A3). In the GOS DP4 fraction, the structures GOS14a or 14b, GOS15, GOS16, GOS17, and GOS18 were found to be monosialylated (Figure 7B2), and the structures GOS14a, 14b, 15, and 16 were found to be disialylated (Figure 7B3). The possible positions of sialic acid ($\alpha 2 \rightarrow 3$)-linked to these GOS compounds are presented in Scheme 3. As expected, the structures GOS6a, GOS9, GOS10a or 10b, GOS14a, GOS14b, GOS15a or 15b, and GOS16a or 16b with two unsubstituted terminal β -Gal residues at O-3 positions were either mono- or disialylated by TcTS.³⁴ The disialylation of the structure GOS6b (with an internal β -Gal residue linked ($\beta 1 \rightarrow 6$) with a terminal β -Gal residue) by this trans-sialidase was already observed in a previous study (Scheme 3).³⁴ The linear Gal($\beta 1 \rightarrow 4$)Gal epitope present in the structures GOS11, 16c, 17, 18a, and 18b allowed only the terminal β -Gal residue to be sialylated by TcTS, resulting in only monosialylation for these types of structures (Scheme 3).

Close inspection of the HPAEC-PAD profiles (Figure 7A) showed in the Vivinal GOS DP3 fraction only trace amounts of GOS12 (3'-galactosyllactose) and a major peak for GOS11 (4'-galactosyllactose). After desialylation of the monosialylated DP3 pool, approximately equal amounts of GOS11 and GOS12 were observed. This fits with observations on LGOS and previous work, showing a higher specificity constant of TcTS toward 3'-galactosyllactose than toward 4'-galactosyllactose.³⁴ Also, in the DP4 fraction (Figure 7B), the linear structures with terminal Gal($\beta 1 \rightarrow 4$) residues (GOS17 and GOS18) showed relatively low peaks, compared with those of the Vivinal GOS DP4 pool, whereas the branched structures had relatively increased.

In this study, the trans-sialidase from *T. cruzi* was used to transfer sialic acid to oligosaccharides (DP3–4) in GL34, LGOS, and Vivinal GOS mixtures.⁸ Decorated structures were identified by HPAEC-PAD chromatography and NMR spectroscopy. As expected, various compounds in these mixtures with one or more accessible β -Gal-OH-3 groups were used as acceptor substrates by TcTS. The F2 (2-Glc-Lac) compound in the GL34 mixture was monosialylated, yielding $\alpha 3$ Sia-2-Glc-Lac with a conversion degree of 47.6%. TcTS was able to transfer sialic acid to at least five different compounds in the LGOS mixture with a conversion degree of up to 52%. The conversion of galacto-oligosaccharides (GOS) with DP3–4 (3 mM GOS with 6 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac) into Sia-GOS by TcTS was clearly lower, at about 35%, but it was obtained under different conditions.⁸ The optimal concentrations of the GL34 and LGOS mixtures for maximal conversion by TcTS ($10 \mu\text{g mL}^{-1}$) in the incubations with 5 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac (from GMP) were 10 and 1 mM, respectively. In fact, all the structures in the LGOS mixture possess terminal nonreducing β -Gal residues, whereas only the F2 compound of the GL34 mixture has a terminal β -Gal residue. Previously, only lactulose was used as an acceptor substrate for a mutant trans-sialidase Tr13 from *T. rangeli*.⁴² The GOS mixture has been known to provide multiple C-3 hydroxyl groups and to be an easily accessible substrate for trans-sialidase, including TcTS (acceptor) sites.^{9,8,43} Our study showed that in fact most of the GOS structures of DP3 and DP4 from Vivinal GOS were sialylated by TcTS. Moreover, the results revealed a strong preference for terminal β -Gal residues to be sialylated. Only branched compounds with two nonreducing terminal β -Gal residues were disialylated. The only exception known so far is 6'-galactosyllactose, which is linear with a specific Gal($\beta 1 \rightarrow 6$)Gal epitope that could be disialylated by TcTS.³⁴ Moreover, our study showed that structures with a Gal($\beta 1 \rightarrow 3$) terminal residues were more efficiently sialylated by TcTS.

In conclusion, the data show that enzymatic synthesis of sialylated lactose- and lactulose-derived oligosaccharides, using the TcTS enzyme and ($\alpha 2 \rightarrow 3$)-Neu5Ac from GMP as a donor substrate, yields a highly interesting variety of sialylated oligosaccharides. This transfer of sialic acid as a functional group is the first step in developing hMOS-mimicking compounds. In future studies, we aim to optimize their biosynthesis and to evaluate the potential use of these novel compounds for pathogen inhibition and prevention of NEC.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06974.

1D ^1H NMR spectrum and HPAEC-PAD profiles of different samples (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

TcTS, trans-sialidase from *Trypanosoma cruzi*; hMOS, human milk oligosaccharides; GOS, galacto-oligosaccharides; GL34, glucosylated-lactose; LGOS, galactosylated-lactulose; Vivinal GOS, galacto-oligosaccharide; GMP, κ -casein-derived glycomacropeptide; HPAEC-PAD, high-pH anion-exchange chromatography–pulse amperometric detection; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; NMR, nuclear magnetic resonance

REFERENCES

- (1) Boehm, G.; Stahl, B. Oligosaccharides from milk. *J. Nutr.* **2007**, *137* (3), 847S–849.
- (2) German, J.; Freeman, S.; Lebrilla, C.; Mills, D. Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria. In *Nestle Nutrition Workshop Series: Paediatric Program*; Nestlé Nutrition Services, 2008; Vol 62, pp 205–218.
- (3) Fuhrer, A.; Sprenger, N.; Kurakevich, E.; Borsig, L.; Chassard, C.; Hennot, T. Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization. *J. Exp. Med.* **2010**, *207* (13), 2843–2854.
- (4) Kurakevich, E.; Hennot, T.; Hausmann, M.; Rogler, G.; Borsig, L. Milk oligosaccharide sialyl(2→3)lactose activates intestinal CD11c⁺ cells through TLR4. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (43), 17444–17449.
- (5) Weiss, G. A.; Hennot, T. The role of milk sialyllactose in intestinal bacterial colonization. *Adv. Nutr.* **2012**, *3* (3), 483S–488S.
- (6) Jantscher-Krenn, E.; Zhrebetsov, M.; Nissan, C.; Goth, K.; Guner, S. Y.; Naidu, N.; Choudhury, B.; Grishin, V. A.; Ford, R. H.; Bode, L. The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotizing enterocolitis in neonatal rats. *Gut* **2012**, *61* (10), 1417–1425.
- (7) Autran, C. A.; Schoterman, M. H. C.; Jantscher-Krenn, E.; Kamerling, J. P.; Bode, L. Sialylated galacto-oligosaccharides and 2'-fucosyllactose reduce necrotizing enterocolitis in neonatal rats. *Br. J. Nutr.* **2016**, *116* (2), 294–299.
- (8) Wilbrink, M. H.; ten Kate, G. a.; Sanders, P.; Gerwig, G. J.; van Leeuwen, S. S.; Sallomons, E.; Klarenbeek, B.; Hage, J. A.; van Vuure, C. A.; Dijkhuizen, L.; Kamerling, J. P. Enzymatic decoration of prebiotic galacto-oligosaccharides (Vivinal GOS) with sialic acid using *Trypanosoma cruzi* trans-Sialidase and two bovine sialoglycoconjugates as donor substrates. *J. Agric. Food Chem.* **2015**, *63* (25), 5976–5984.
- (9) Holck, J.; Larsen, D. M.; Michalak, M.; Li, H.; Kjærulff, L.; Kirpekar, F.; Gotfredsen, C. H.; Forssten, S.; Ouwehand, A. C.; Mikkelsen, J. D.; Meyer, A. S. Enzyme catalysed production of sialylated human milk oligosaccharides and galacto-oligosaccharides by *Trypanosoma cruzi* trans-sialidase. *New Biotechnol.* **2014**, *31* (2), 156–165.
- (10) Idota, T.; Kawakami, H.; Murakami, Y.; Sugawara, M. Inhibition of cholera toxin by human milk fractions and sialyllactose. *Biosci., Biotechnol., Biochem.* **1995**, *59* (3), 417–419.
- (11) Ruiz-Palacios, G. M.; Cervantes, L. E.; Ramos, P.; Chavez-Munguia, B.; Newburg, D. S. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuca1, 2Galβ1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J. Biol. Chem.* **2003**, *278* (16), 14112–14120.
- (12) Andersson, B.; Porras, O.; Hanson, L. A.; Lagergård, T.; Svanborg-Edén, C. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. *J. Infect. Dis.* **1986**, *153* (2), 232–237.
- (13) Holmgren, J.; Svennerholm, A. M.; Lindblad, M. Receptor-like glycomolecules in human milk that inhibit classical and El Tor *Vibrio cholerae* cell adherence (Hemagglutination). *Infect. Immun.* **1983**, *39* (1), 147–154.
- (14) Parkkinen, J.; Finne, J.; Achtman, M.; Väisänen, V.; Korhonen, T. K. *Escherichia coli* strains binding neuraminyl ($\alpha 2 \rightarrow 3$) galactosides. *Biochem. Biophys. Res. Commun.* **1983**, *111* (2), 456–461.
- (15) Urashima, T.; Saito, T.; Nakamura, T.; Messer, M. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj. J.* **2001**, *18*, 357–371.
- (16) Gopal, P. K.; Gill, H. S. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br. J. Nutr.* **2000**, *84* (S1), 69–71.
- (17) Albrecht, S.; Lane, J. A.; Mariño, K.; Al Busadah, K.; Carrington, S. D.; Hickey, R. M.; Rudd, P. M. A comparative study of free oligosaccharides in the milk of domestic animals. *Br. J. Nutr.* **2014**, *111* (7), 1313–1328.
- (18) Boehm, G. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Arch Dis Child - Fetal Neonatal Ed.* **2002**, *86* (3), 178F–181.
- (19) Van Leusen, E.; Torringa, E.; Groenink, P.; Kortleve, P.; Geene, R.; Schoterman, M.; Klarenbeek, B. Industrial applications of galacto-oligosaccharides. In *Food Oligosaccharides: Production, Analysis and Bioactivity*; Wiley, 2014; pp 470–491.
- (20) Pham, T. T. H.; Dijkhuizen, L.; van Leeuwen, S. S. Structural characterization of glucosylated lactose derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes. *Carbohydr. Res.* **2017**, *449*, 59–64.
- (21) Yin, H.; Dijkhuizen, L.; van Leeuwen, S. S. Synthesis of galacto-oligosaccharides derived from lactulose by wild-type and mutant β -galactosidase enzymes from *Bacillus circulans* ATCC 31382. *Carbohydr. Res.* **2018**, *465*, 58–65.
- (22) Pham, T. T. H.; Böger, C. L. M.; Dijkhuizen, L.; van Leeuwen, S. S. Stimulatory effects of novel glucosylated lactose derivatives GL34 on growth of gut bacteria. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 707–718.
- (23) Cardelle-Cobas, A.; Fernández, M.; Salazar, N.; Martínez-Villaluenga, C.; Villamiel, M.; Ruas-Madiedo, P.; de los Reyes-Gavilán, C. G. Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. *J. Dairy Res.* **2009**, *76* (3), 317–325.
- (24) Algieri, F.; Rodríguez-Nogales, A.; Garrido-Mesa, N.; Vezza, T.; Garrido-Mesa, J.; Utrilla, M. P.; Montilla, A.; Cardelle-Cobas, A.; Olano, A.; Corzo, N.; Guerra-Hernandez, E.; Zarzuelo, A.; Rodríguez-Cabezas, M. E.; Galvez, J. Intestinal anti-inflammatory effects of oligosaccharides derived from lactulose in the trinitrobenzenesulfonic acid model of rat colitis. *J. Agric. Food Chem.* **2014**, *62* (19), 4285–4297.
- (25) Marín-Manzano, M. C.; Abecia, L.; Hernández-Hernández, O.; Sanz, M. L.; Montilla, A.; Olano, A.; Rubio, L. A.; Moreno, F. J.; Clemente, A. Galacto-oligosaccharides derived from lactulose exert a selective stimulation on the growth of *Bifidobacterium animalis* in the large intestine of growing rats. *J. Agric. Food Chem.* **2013**, *61* (31), 7560–7567.
- (26) Laparra, J. M.; Hernandez-Hernandez, O.; Moreno, F. J.; Sanz, Y. Neoglycoconjugates of caseinomacropeptide and galacto-oligosaccharides modify adhesion of intestinal pathogens and inflammatory response(s) of intestinal (Caco-2) cells. *Food Res. Int.* **2013**, *54* (1), 1096–1102.
- (27) Schauer, R.; Kamerling, J. P. The chemistry and biology of trypanosomal trans-Sialidases: Virulence factors in chagas disease and sleeping sickness. *ChemBioChem* **2011**, *12* (15), 2246–2264.
- (28) Schenkman, S.; Eichinger, D. *Trypanosoma cruzi* trans-sialidase and cell invasion. *Parasitol. Today* **1993**, *9* (6), 218–222.
- (29) Schenkman, S.; Ferguson, M. A. J.; Heise, N.; Cardoso de Almeida, M. L.; Mortara, R. A.; Yoshida, N. Mucin-like glycoproteins

linked to the membrane by glycosylphosphatidylinositol anchor are the major acceptors of sialic acid in a reaction catalyzed by *trans*-sialidase in metacyclic forms of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **1993**, *59* (2), 293–303.

(30) Amaya, M. F.; Watts, A. G.; Damager, I.; Wehenkel, A.; Nguyen, T.; Buschiazzi, A.; Paris, G.; Frasch, A. C.; Withers, S. G.; Alzari, P. M. Structural insights into the catalytic mechanism of *Trypanosoma cruzi trans*-sialidase. *Structure* **2004**, *12* (5), 775–784.

(31) Watts, A. G.; Damager, I.; Amaya, M. L.; Buschiazzi, A.; Alzari, P.; Frasch, A. C.; Withers, S. G. *Trypanosoma cruzi trans*-sialidase operates through a covalent sialyl-enzyme intermediate: Tyrosine is the catalytic nucleophile. *J. Am. Chem. Soc.* **2003**, *125* (25), 7532–7533.

(32) Buschiazzi, A.; Amaya, M. F.; Cremona, M. L.; Frasch, A. C.; Alzari, P. M. The crystal structure and mode of action of *trans*-sialidase, a key enzyme in *Trypanosoma cruzi* pathogenesis. *Mol. Cell* **2002**, *10* (4), 757–768.

(33) Vandekerckhove, F.; Schenkman, S.; de Carvalho, L. P.; Tomlinson, S.; Kiso, M.; Yoshida, M.; Hasegawa, A.; Nussenzweig, V. Substrate specificity of the *Trypanosoma cruzi trans*-sialidase. *Glycobiology* **1992**, *2* (6), 541–548.

(34) Wilbrink, M. H.; ten Kate, G. a.; van Leeuwen, S. S.; Sanders, P.; Sallomons, E.; Hage, J. A.; Dijkhuizen, L.; Kamerling, J. P. Sialylation of Galactosyl-lactose using *Trypanosoma cruzi trans*-sialidase as the biocatalyst and bovine casein derived glycomacropeptide as the donor substrate. *Appl. Environ. Microbiol.* **2014**, *80* (19), 5984–5991.

(35) Holland, J. W.; Deeth, H. C.; Alewood, P. F. Analysis of O-glycosylation site occupancy in bovine κ -casein glycoforms separated by two-dimensional gel electrophoresis. In: *Proteomics* **2005**, *5*, 990–1002.

(36) Van Halbeek, H.; Dorland, L.; Vliegthart, J. F. G.; Fiat, A. M.; Jolles, P. A 360-MHz ^1H -NMR study of three oligosaccharides isolated from cow κ -casein. *Biochim. Biophys. Acta, Protein Struct.* **1980**, *623* (2), 295–300.

(37) Buschiazzi, A.; Campetella, O.; Frasch, A. C. C. *Trypanosoma rangeli* sialidase: cloning, expression and similarity to *T. cruzi trans*-sialidase. *Glycobiology* **1997**, *7* (8), 1167–1173.

(38) Ribeiro, M.; Pereira-Chioccola, V. L.; Eichinger, D.; Rodrigues, M. M.; Schenkman, S. Temperature differences for *trans*-glycosylation and hydrolysis reaction reveal an acceptor binding site in the catalytic mechanism of *Trypanosoma cruzi trans*-sialidase. *Glycobiology* **1997**, *7* (8), 1237–1246.

(39) Vliegthart, J. F. G.; Kamerling, J. P. ^1H NMR structural-reporter-group concepts in carbohydrate analysis. *Compr. Glycosci. From Chem. to Syst. Biol.* **2007**, *2*, 133–191.

(40) Van Leeuwen, S. S.; Kuipers, B. J. H.; Dijkhuizen, L.; Kamerling, J. P. Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products. *Carbohydr. Res.* **2016**, *425*, 48–58.

(41) van Leeuwen, S. S.; Leeftang, B. R.; Gerwig, G. J.; Kamerling, J. P. Development of a ^1H NMR structural-reporter-group concept for the analysis of prebiotic galacto-oligosaccharides of the $[\beta\text{-d-Galp-(1}\rightarrow\text{x)]n\text{-d-Glcp}$ type. *Carbohydr. Res.* **2008**, *343* (6), 1114–1119.

(42) Jers, C.; Michalak, M.; Larsen, D. M.; Kepp, K. P.; Li, H.; Guo, Y.; Kirpekar, F.; Meyer, A. S.; Mikkelsen, J. Rational design of a new *Trypanosoma rangeli trans*-sialidase for efficient sialylation of glycans. *PLoS One* **2014**, *9* (1), e83902.

(43) Zeuner, B.; Holck, J.; Perna, V.; Mikkelsen, J. D.; Meyer, A. S. Quantitative enzymatic production of sialylated galacto-oligosaccharides with an engineered sialidase from *Trypanosoma rangeli*. *Enzyme Microb. Technol.* **2016**, *82*, 42–50.